Analysis of Tocopherols by Capillary Supercritical Fluid Chromatography and Mass Spectrometry[1]

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Tocopherol-containing mixtures were analyzed by gas chromatography (GC) and capillary supercritical fluid chromatography (SFC). GC analysis of tocopherols required the formation of the silyl derivatives, while SFC analysis of the tocopherol-containing mixtures was accomplished on neat samples. SFC analysis conditions were optimized with respect to column type and density/pressure programming. Enhanced resolution of many components was achieved by using inverse temperature programming during the SFC analyses. Both SFC and GC analyses permitted the separation and quantitation of alpha-, beta-, gamma- and delta-tocopherols. In addition, SFC proved particularly applicable for characterizing the composition of a deodorizer distillate and commercial antioxidant formulation. Coupling of a quadrapole mass spectrometer with a supercritical fluid chromatograph was also achieved; the mass spectrometer provided electron impact mass spectra on the underivatized tocopherol and sterol moieties. Both SFC and SFC/mass spectrometry proved effective for the analysis of complex lipid-containing mixtures, requiring minimal sample preparation prior to analysis.

KEY WORDS: Chromatography, mass spectrometry, sterol, supercritical fluid, tocopherol.

Analysis of tocopherol-containing mixtures has been traditionally accomplished by thin-layer chromatography (TLC) and gas chromatography (GC), and more recently, by high-performance liquid chromatography (HPLC). Gas-chromatographic procedures usually require derivatization of the tocopherols by silylation (1-4) or acylation (5) to form more volatile species that are amenable to GC. Tocopherol analysis by HPLC can be accomplished by normal-phase (6-9) or reverse-phase techniques (10-13) with ultraviolet or fluorescent detection. The HPLC methods are sensitive to trace levels of tocopherols, but provide limited information on other components that occur with the tocopherols in lipid mixtures (14).

Several researchers (15,16) have demonstrated that tocopherols can be chromatographed and separated from other lipid components by supercritical fluid chromatography (SFC). Although SFC has existed for over two decades (17), the technique has experienced a renaissance only recently, due to improvements in instrumentation and column technology (18). SFC offers some unique advantages to the oil/fat analyst because it permits direct injection and separation of lipophilic mixtures with molecular weights ranging from 100-1000 amu. When coupled with flame-ionization detection, SFC allows the quantitation of many lipid species that cannot be determined by GC and HPLC methods.

Tocopherol-containing mixtures are frequently sold as items of commerce (19) or occur in various by-products of the oil refining process (14,20). For this reason, there is interest not only in determining the tocopherol content of such mixtures but also in the identity and amount of contaminant species. In addition, new isolation methods, *e.g.*, supercritical fluid extraction (SFE), for enriching tocopherols from naturally occurring materials require a detailed analytical analysis of the extract to allow optimization of the extraction conditions (6,21-23). The partial analysis of such mixtures by GC has been reported in the literature (4,6,24,25).

SFC techniques are applicable to these analysis problems and offer considerable analytical versatility in characterizing complex lipid mixtures. In this study, characterizations of the tocopherol content of a commercial antioxidant formulation and a deodorizer distillate were determined by both SFC and GC. In addition, a benchtop quadrapole mass spectrometer (MS) was coupled to the supercritical fluid chromatograph to permit identification of the SFC chromatographic tocopherol peaks.

EXPERIMENTAL PROCEDURES

Materials. Alpha- and delta-tocopherol standards were purchased from the Aldrich Chemical Co. (Milwaukee, WI), while beta- and gamma-tocopherols were obtained from Matreya, Inc. (Pleasant Gap, PA). The gas-chromatographic derivatization agent, Syloon BFT (99 parts of *bis*(trimethylsilyl)trifluoracetamide to 1 part trimethylchlorosilane), was obtained from Supelco Inc. (Bellefonte, PA). Antioxidant samples (Coviox T-50) were obtained from the Henkel Corp. (Hoboken, NJ), and the deodorizer distillate was from a soybean oil processing company.

Three mixtures of pure tocopherol compounds were prepared to compare the SFC with the GC analysis. One mixture contained approximately equal amounts of each tocopherol. Another mixture was formulated to simulate the relative amount of the four tocopherols occurring in soybean oil. The third mixture was made up to contain different amounts of each tocopherol. Each tocopherol mixture, the deodorizer distillate and the antioxidant sample were analyzed five times each by both SFC and GC methods to ascertain the reproducibility of each method.

Chromatographic analysis. SFC analyses of tocopherol standards and lipid mixtures were accomplished with a Lee Scientific Model 601 supercritical fluid chromatograph (Dionex Corp., Salt Lake City, UT). SFC-grade carbon dioxide (Air Products, Inc., Allentown, PA) was used as the carrier fluid. SB-Methyl, SB-Octyl-50, SB-Phenyl-5 and Carbowax capillary columns (100 μm $\times$ 10 m), obtained from Dionex Corporation, were used in this study. Tocopherols and other solutes were detected and quantitated by a flame-ionization detector (FID) held at 350°C. Samples were diluted in hexane (a range of 0.1-1.0 wt%

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dilutions) and were loaded into the sample loop of the injector valve. Two-hundred-nanoliter samples were injected onto the column. Quantitation of the chromatographic data was accomplished by using a Spectra Physics DataJet integrator (Fremont, CA).

Several combinations of pressure/density and temperature programs were explored to optimize the separation of tocopherols and related lipid components. Although many of these programs were adequate for the separation of the tocopherols, the following program provided optimal separation of the tocopherols and other lipid components on the SB-Octyl-50 column. In this pressure program, the initial pressure was held at 100 atm for 10 min and was then increased to 180 atm at the rate of 5 atm/min. The ramp rate at this point was then changed to 2 atm/min until a pressure of 220 atm was reached. The pressure programming rate was then changed to 5 atm/min until a pressure of 350 atm was reached. It was also found that superimposition of an oven temperature gradient program concurrently with the pressure program maximized the resolution between the tocopherols. The temperature program consisted of holding the column oven temperature at 100°C for 10 min, then ramping to 180°C at a rate of 5°C/min and then inversely programming the oven temperature from 180 to 100°C at a rate of −5°C/min.

For GC analysis, the tocopherol standards and samples were derivatized by the method of Marks (3). A range of tocopherol standards (10–20 mg) were weighed into an Erlenmeyer flask, and 0.5 mL pyridine and 1 mL of Sylon BFT were added to the standard. This mixture was then heated at 50°C for 10 min and cooled. Then 5 mL of chloroform, along with an internal standard methyl stearate (15 mg) (Nu-Chek-Prep, Inc., Elysian, MN), were added. Likewise, 100-mg samples of deodorizer distillate and commercial antioxidant sample were prepared for analysis by the same silylation procedure.

Gas-chromatographic analysis was accomplished on a Varian Model 3700 gas chromatograph (Varian Instruments Div., Walnut Creek, CA) using FID. The injector and detector temperatures were 210 and 290°C, respectively. A DB-5 capillary column, 0.32 mm × 30 m × 1 μm film thickness (J&W Scientific, Folsom, CA), was used to effect the separation of tocopherol derivatives. The GC oven temperature was initially held at 140°C for one minute and then programmed from 140 to 290°C at 5°C/min to obtain the desired separation. A 2-μL sample of the above derivatized mixtures was injected onto the GC column. Split injection, with a 50:1 split, was used; helium velocity was 28 cm/s at 100°C.

The SFC unit was also interfaced with a Finnigan Incos 50 mass spectrometer (Finnigan Corp., San Jose, CA) to confirm the identity of the tocopherol compounds and other components present in the deodorizer distillate and antioxidant sample. This required minor modification of the SFC and MS units for the interface installation, but considerable alteration of a commercial interface purchased from the Dionex Corporation. The commercial interface was shortened by removing the elbow portion of the heated transfer line to permit a more direct transfer of the chromatographed sample into the ionization source of the mass spectrometer. This step required vertically aligning the SFC unit with respect to the MS. The fused-silica transfer capillary was then encased in copper powder (J.T. Baker, Inc., Phillipsburg, NJ) and sealed in a copper tube for better temperature control of the transfer line. Additional cartridge heating elements (Finnigan Corp.) were also added, along with a thermocouple probe and readout, to assure more even heating of the transfer line. The transfer line temperature was maintained at 12°C; the probe temperature was 300°C and the source temperature was 200°C.

SFC with mass-spectrometric detection was similar to that for the SFC runs. The SB-Octyl-50 capillary column was again used to separate the tocopherols and related components, as well as to allow a comparison of the SFC/MS results with other chromatographic data. However, a density-based program was used in some cases for the SFC/MS analyses. It was found that a density program from 0.20 g/mL to 0.66 g/mL at programming rate of 0.002 g/mL/min produced an excellent separation of the tocopherols and sterols at a column temperature of 120°C.

RESULTS AND DISCUSSION

Initial SFC studies were conducted to determine the retention characteristics of the tocopherol moieties on different capillary column stationary phases. Both a SB-Phenyl-5 and a SB-Octyl-50 column were evaluated with respect to the relative retention and separation of the four tocopherols. Initial experiments on the SB-Phenyl-5 column showed that a column temperature in excess of 100°C was necessary for tocopherol elution. SFC runs on these nonpolar stationary phases indicated that the analyte elution order was delta-tocopherol followed by beta-, gamma- and alpha-tocopherol. All of the tocopherol peaks were found to elute between 200–240 atm. Additional experiments with different pressure programming rates and initial isobaric holds provided only partial separation of the beta-tocopherol from the gamma isomer. The resolution between these two isomers was somewhat improved on the SB-Octyl-50 column. For this reason, the SB-Octyl-50 column was chosen for additional SFC and SFC/MS studies. Retention times on the octyl and phenyl stationary phases for the tocopherol peaks appear to follow a molecular weight dependence, *i.e.*, the addition of methyl groups on the aromatic ring of the tocopherols increases the retention time. Common phytosterols, which also occur in tocopherol-containing mixtures, elute after the tocopherol peaks on the nonpolar SFC columns. This trend is readily apparent in the SFC runs illustrated in Figures 1 and 2.

However, this elution order is reversed when SFC is attempted on the Carbowax bonded column. As shown in Figure 3, the phytosterol peaks elute before the tocopherols on the Carbowax column. Furthermore, the elution order of the individual tocopherols is reversed from that observed on the SB-octyl-50 stationary phase. The tocopherol elution order shown in Figure 3 can be partially rationalized on the basis of a steric inhibition effect, which limits interaction between the hydroxyl group in the tocopherol moiety and the ether and hydroxyl functionalities of the polar Carbowax stationary phase. As indicated by the structures in Figure 4, the alpha-tocopherol molecule has two methyl groups alpha to the hydroxyl group on the aromatic ring, thereby inhibiting interaction with the polar stationary phase. This results in the alpha-tocopherol having the shortest retention time of three tocopherols chromatographed in Figure 3.

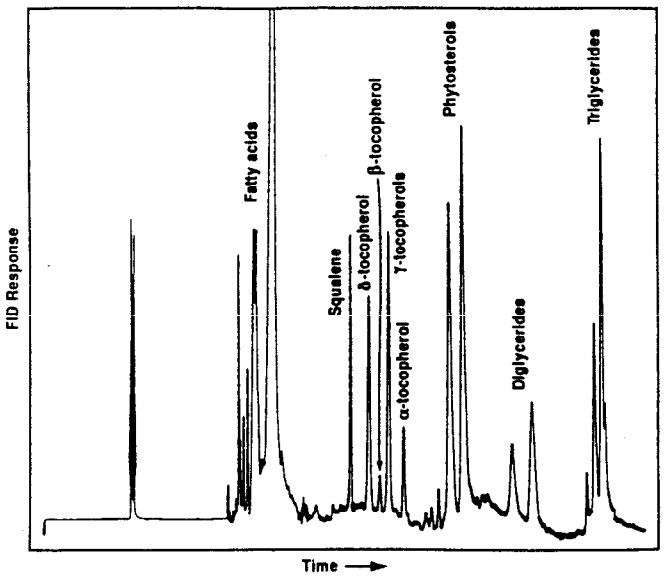

FIG. 1. Supercritical fluid chromatography analysis of a deodorizer distillate with the SB-Octyl-50 column. FID, flame-ionization detector.

Gamma-tocopherol, with only one methyl group alpha to the hydroxyl moiety, is next to elute. It is followed by the delta-tocopherol which is devoid of any steric inhibition around the hydroxyl group.

The chromatogram of the deodorizer distillate shown in Figure 1 illustrates the versatility of SFC. Deodorizer distillate is a complex mixture of many lipophilic components. Both tocopherols and sterols are present in the distillate which is used as a feedstock for preparation of sterol and tocopherol concentrates (14). Appreciable amounts of other components may also be present in the distillate, such as stearic or oleic acids, as well as hydrocarbons, predominately squalane or squalene. High-molecular weight species, such as di- or triglycerides, may also be carried over in the distillate, making the analysis of such mixtures difficult.

Figure 1 shows that most of these species can be separated and quantitated by SFC. The illustrated separation was accomplished under the conditions reported in the Experimental Procedures section as optimized for separation of the tocopherol compounds. The separation of other components in the deodorizer distillate can be achieved by altering the pressure programming parameters, but the described program is effective in separating many major components in the distillate. Both squalane and squalene are readily separated on this column under the reported chromatographic conditions. The fatty acids, present in the deodorizer distillate, can be resolved with a different pressure program; however, the nonpolar capillary column is sensitive to sample overload. The deodorizer distillate in the cited case was diluted in hexane and analyzed without any prior sample preparation before injection.

The SFC/MS analysis of a commercial antioxidant mixture is shown in Figure 2. The total ion chromatogram determined from the MS in the electron ionization (EI) mode clearly indicates the separation of the tocopherols and sterols. The individual peaks in Figure 2 were identified by comparing their retention times and EI mass spectra with those of individual tocopherol standards. The resultant mass spectra are shown in Figure 5 for three of the individual tocopherols and for the unknown peak eluting after squalene in Figure 2. All of them showed excellent matches between the standard compounds and the identified peaks in Figure 2, shown by the corresponding spectra for delta-tocopherol standard (Fig. 5c) and the delta-tocopherol (Fig. 5d) in the antioxidant sample.

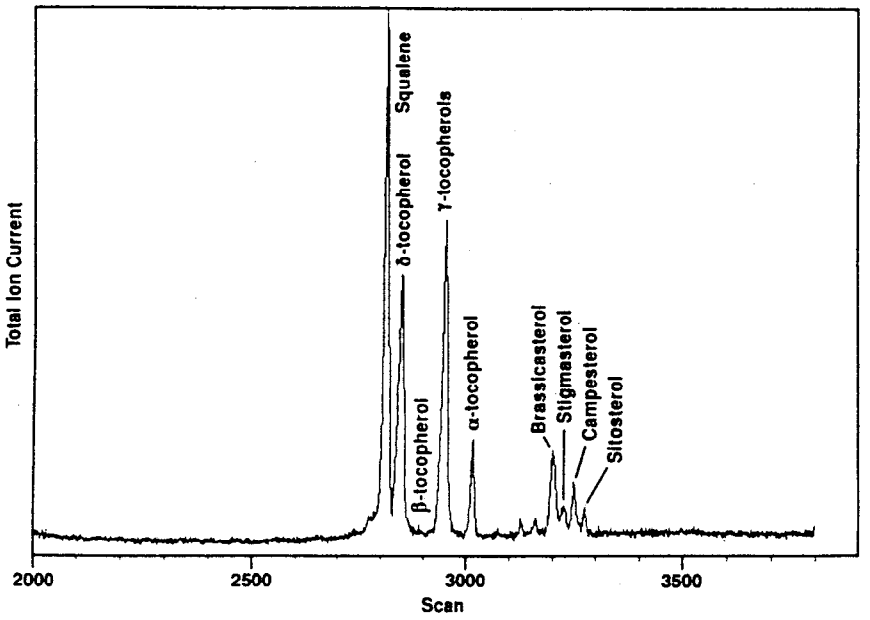

FIG. 2. Supercritical fluid chromatography/mass spectrometry analysis of a commercial antioxidant sample with the SB-Octyl-50 column.

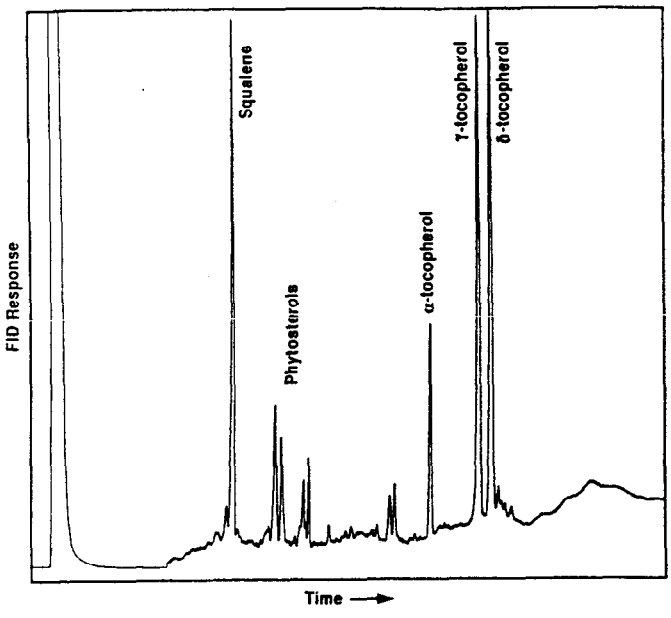

FIG. 3. Supercritical fluid chromatography analysis of a commercial antioxidant sample with the Carbowax column. FID, flame-ionization detector.

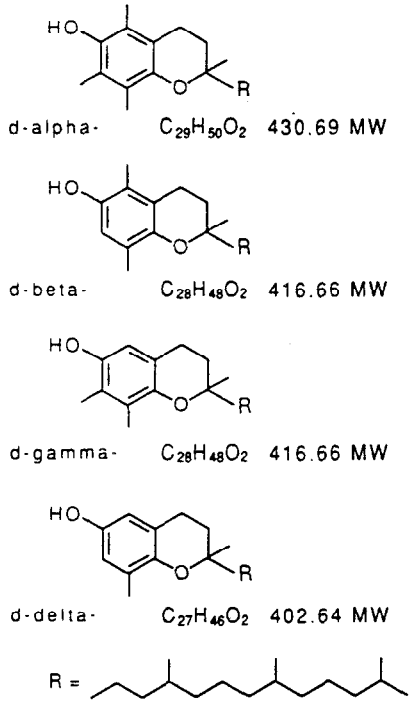

FIG. 4. Tocopherol structures.

The EI mass spectra shown in Figure 5 were determined on underivatized compounds and appear to be the only mass spectra determined to date on neat tocopherols. Unlike GC/MS studies, where derivatized compounds are often required, SFC/MS extends the range of compounds that can be chromatographed with subsequent MS identification on the underivatized compound. Prior GC/MS studies (26) have established EI/MS spectra for the trifluoroacetate derivative of the parent tocopherols. The fragmentation patterns observed in the SFC/MS case strongly resemble the cited GC/MS spectra. For example, the mass spectrm for alpha-tocopherol in Figure 5a shows a small but identifiable molecular ion at $m/e = 430$. In addition, there is evidence for the loss of the side chain from the chroman ring at $m/e = 205$. A strong base ion peak is apparent at $m/e = 165$, resulting from cleavage and hydrogen migration at the heterocyclic chroman ring.

The SFC/MS-derived mass spectrum for gamma-tocopherol (Fig. 5b) also matches well with the GC/MS-derived mass spectrum, showing the appearance of a molecular ion at $m/e = 416$ and major fragment ions at m/e of 151 and 191. The formation of these later ions has been postulated by Elliott and Waller (26), and a similar dissociative mechanism seems feasible for the SFC/MS case.

For delta-tocopherol (Fig. 5c), the SFC/MS-derived spectrum indicates the appearance of a molecular ion at $m/e = 402$ and fragment ions at m/e of 177 and 137. The 40 m/e interval suggests a similar ionization pattern to that postulated for alpha- and beta-tocopherol. As noted previously, the delta-tocopherol spectrum is identical to the spectrum (Fig. 5d) shown by one of the peaks in the total ion current chromatogram in Figure 2.

Under similar SFC/MS conditions, other commercial tocopherol-containing antioxidant formulations can be analyzed and their components identified. In the course of these studies, vitamin E was separated from its alpha-tocopherol precursor. Formulations and mixtures containing triglyceride-based oils, such as soybean and wheat germ, were also analyzed by SFC/MS; however, resolution of the triglycerides was lost through the above-described interface, indicating the necessity to improve the transmission of these higher-molecular weight species through the interface.

Quantitative SFC and GC studies on the three previously described tocopherol standard mixtures are reported in Table 1. The agreement is good between the results of the SFC and GC analysis when compared to the gravimetrically weighed standard mixtures. The reproducibilities of the SFC and GC analysis are comparable as judged by the percent relative standard deviations, ranging between 1-5%, as determined for five individual analysis.

Similarly, tocopherol concentrations were determined in an antioxidant sample and in deodorizer distillate as shown in Table 2. In this data set there are some differences between the SFC and GC results, despite the fact that both techniques yielded similar relative standard deviations. The SFC analysis consistently gave higher values than the GC results for the tocopherol content of these mixtures. Such a trend could be due to loss of analyte in the GC procedure, due to the necessity to derivatize the tocopherols prior to chromatography.

In summary, SFC analysis of tocopherols offers several unique features to the analyst: no need for sample preparation prior to analysis, avoidance of sample derivatization of nonvolatile species and the ability to adjust elution conditions to analyze higher-molecular weight components that may be present in the sample. Likewise, SFC/MS appears to be particularly well suited for rapid analysis and identification of tocopherols and associated lipid species,

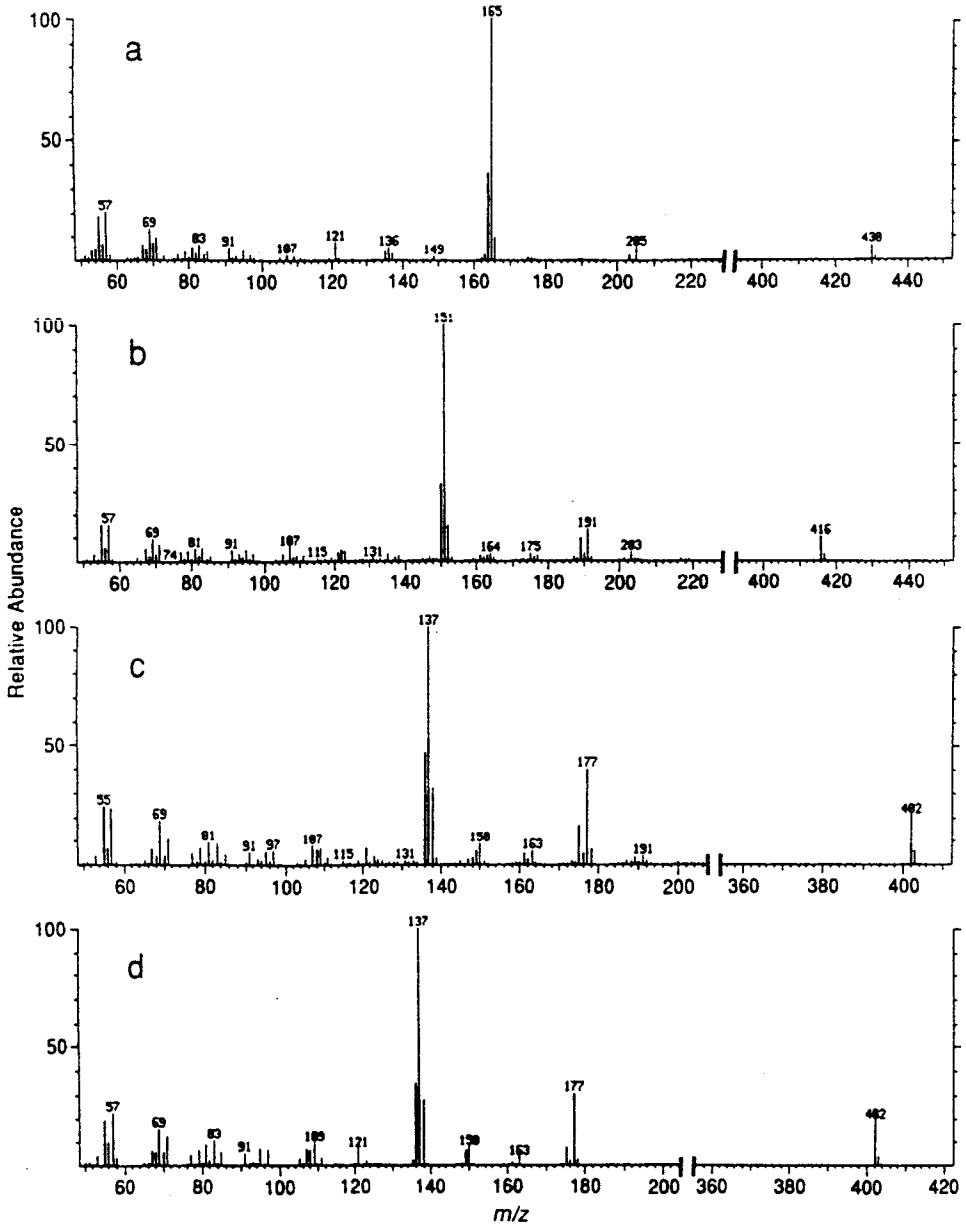

FIG. 5. Electron ionization mass spectra derived from supercritical fluid chromatography/mass spectrometry; (a) α-tocopherol standard; (b) γ-tocopherol standard; (c) δ-tocopherol standard; (d) δ-tocopherol in commercial antioxidant sample.

yielding mass spectra equivalent to those obtained by conventional GC/MS. Quantitative studies have shown that SFC is capable of determining the concentration of tocopherols in commercial antioxidant and process samples.

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TABLE 1

Mixtures of Tocopherol Standards

Tocopherol	Concentration (mg/100 mg)		
	Weight	SFC analysis[a]	GC analysis[a]
Sample 1			
alpha	10.0	9.1(2.14)[b]	11.1(2.30)[b]
beta	10.1	11.0(2.95)	9.8(3.88)
gamma	9.9	10.2(4.17)	12.1(2.79)
delta	11.0	9.1(2.82)	11.1(2.05)
Sample 2			
alpha	20.1	21.3(3.50)	20.9(1.15)
beta	4.1	4.0(3.69)	5.0(4.3)
gamma	23.0	23.1(2.01)	26.2(1.12)
delta	25.9	26.3(4.37)	23.4(1.96)
Sample 3			
alpha	15.0	16.4(4.34)	13.4(5.11)
beta	25.8	22.3(2.65)	24.2(3.22)
gamma	41.2	39.2(2.84)	38.0(3.99)
delta	36.4	37.8(2.77)	34.2(2.84)

[a] Average of five analyses. Abbreviations: SFC, supercritical fluid chromatography; GC, gas chromatography.

[b] The numbers in parentheses are relative standard deviations.

TABLE 2

Tocopherol Concentration in Commercial Samples

	Deodorizer distillate		Antioxidant sample	
	SFC Analysis[a] mg/100 mg	GC Analysis[a] mg/100 mg	SFC Analysis[a] mg/100 mg	GC Analysis[a] mg/100 mg
Alpha	2.37(6.95)[b]	1.59(3.62)	7.21(2.04)	8.98(1.54)
Beta	0.58(7.55)	0.36(10.89)	0.52(20.39)	0.24(21.15)
Gamma	8.56(4.20)	7.14(3.32)	23.47(2.49)	22.39(1.46)
Delta	6.24(2.68)	4.89(5.62)	25.24(1.79)	24.49(1.90)

[a] Average of five analyses. Abbreviations: SFC, supercritical fluid chromatography; GC, gas chromatography.

[b] The numbers in parentheses are relative standard deviations.

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